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Review

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Glufosinate-ammonium: a review of the current state of knowledge

Hudson K Takano* and Franck E Dayan

Abstract

Glufosinate is a key herbicide to manage glyphosate-resistant weeds mainly because it is a broad-spectrum herbicide, and transgenic glufosinate-resistant crops are available. Although glufosinate use has increased exponentially over the past decade, the treated area with this herbicide is far less than that with glyphosate. This is because glufosinate often provides inconsistent performance in the field, which is attributed to several factors including environmental conditions, application technology, and weed species. Glufosinate is also highly hydrophilic and does not translocate well in plants, generally providing poor control of grasses and perennial species. In the soil, glufosinate is rapidly degraded by microorganisms, leaving no residual activity. While there have been concerns regarding glufosinate toxicology, its proper use can be considered safe. Glufosinate is a fast-acting herbicide that was first discovered as a natural product, and is the only herbicide presently targeting glutamine synthetase. The mode of action of glufosinate has been controversial, and the causes for the rapid phytotoxicity have often been attributed to ammonia accumulation. Recent studies indicate that the contact activity of glufosinate results from the accumulation of reactive oxygen species and subsequent lipid peroxidation. Glufosinate disrupts both photorespiration and the light reactions of photosynthesis, leading to photoreduction of molecular oxygen, which generates reactive oxygen species. The new understanding of the mode of action provided new ideas to improve the herbicidal activity of glufosinate. Finally, a very few weed species have evolved glufosinate resistance in the field, and the resistance mechanisms are generally not well understood requiring further investigation.
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Supporting information may be found in the online version of this article.

Keywords: phosphinothricin; glutamine synthetase; reactive oxygen species; mode of action; herbicide resistance; genetically modified crops; glufosinate

1 INTRODUCTION

Glufosinate-ammonium (D,L-phosphinothricin or 2-amino-4-(hydroxymethylphosphinyl)butanoic acid) is a herbicide with a number of unique characteristics that have intrigued researchers for many years across different scientific areas. Glufosinate was discovered as a natural product once characterized as 'a natural amino acid with unexpected herbicidal properties'.[1] Glufosinate has an unusual physicochemical profile compared to the majority of herbicides currently used in the world, and is the most important herbicide targeting glutamine synthetase (GS).[2] The mode of action of glufosinate has been very controversial for almost 30 years since its first introduction in 1993. Furthermore, environmental conditions can strongly affect glufosinate performance in the field, and only a few weed species have evolved glufosinate resistance in the world. In this review, we discuss the most fascinating aspects of glufosinate as a herbicide, from its first discovery to the complete mode-of-action elucidation and weed resistance evolution.

2 DISCOVERY AND COMMERCIALIZATION

Glufosinate was first discovered by two different research groups investigating the actinomycetes *Streptomyces hygroscopicus* and *S. viridochromogenes*.[3] Under fermentation culture conditions, these microorganisms can produce a tripeptide called bialaphos (4-[hydroxy(methyl)phosphinoyl]-L-homoalanyl-L-alanyl-L-alanine) (Fig. 1). The synthesis of bialaphos is not the focus of our manuscript, but it has been recently summarized in a comprehensive review.[4] The entire process requires 18 steps from the building block phosphoenolpyruvate to the end product bialaphos. Bialaphos is currently used in eastern Asia as a broad-spectrum and non-selective herbicide. Once inside the plant, the tripeptide is metabolized through hydrolytic cleavage of the two alanine residues, releasing L-phosphinothricin, the actual phytotoxic molecule.[2]

Glufosinate is formulated as a racemic mixture of D, L-phosphinothricin under different commercial brands. It is often referred to as glufosinate-ammonium because the ammonium-salt formulations are the most commonly used. At least five different chemical methods have been developed for the enantioselective synthesis of L-phosphinothricin.[4] However, the cost to produce field scale amounts of the pure L-isomer still remains substantially higher than that for the racemic mixture. However, a simpler method to convert D-phosphinothricin into L-phosphinothricin has also been proposed, providing an

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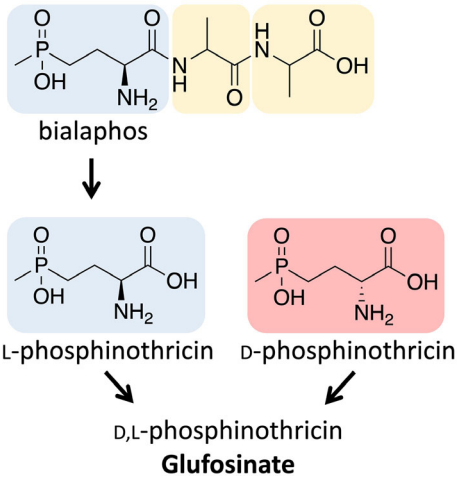

Figure 1 Bialaphos, a tripeptide (L-phosphinothricin + L-alanine + L-alanine), is naturally produced by *Streptomyces* sp. and metabolically bioactivated into L-phosphinothricin by plants. Glufosinate is currently formulated as a racemic mixture of D, L-phosphinothricin.

increased proportion of the active enantiomer (Fig. 2).[5] The latter is a fairly simple process involving a two-step reaction: oxidation of the D-isomer into PPOB (4-methylphosphinico-2-oxo-butanoic acid), followed by a transamination or amino transferase yielding L-phosphinothricin. The conversion of PPOB into L-phosphinothricin can also be performed enzymatically through glutamate dehydrogenase, with optimized affinity for glufosinate.[6] More recently, another method has been proposed to increase the proportion of L-phosphinothricin at up to 96% by coupling the amidase-mediated hydrolysis of *N*-phenylacetyl-D,L-phosphinothricin with racemization of *N*-phenylacetyl-D-phosphinothricin.[7] Field and laboratory experiments demonstrated that formulations with increased proportions of L-phosphinothricin provided similar levels of both grass and broadleaf weed control as well as lower crop injury for cotton and soybean compared to equivalent amounts of the L-isomer in the racemic mixture.[5]

Glufosinate was first commercialized in the United States and Canada in 1993–1994 as a non-selective herbicide with broad spectrum of weed control. The estimated treated area with glufosinate in the world was approximately 12 million hectares per year in 2014,[9] following a substantial increase in the United States over the past decade (Fig. 3(a)). The main reason for this increase in use was to combat the ever increasing number of glyphosate-resistant weeds, as glufosinate works as an alternative herbicide to glyphosate, the most popular herbicide in the world. In addition, glufosinate-resistant crops were rarely used before glyphosate-resistant weeds became a major problem in glyphosate-resistant crops, even though the two technologies became available around the same time. This is probably because glufosinate does not provide the same level of efficacy and consistency as glyphosate for numerous reasons that will be discussed later in this manuscript. Thus, glufosinate is widely used in the Midwest and Southern United States where the majority of glufosinate-resistant soybean and cotton are planted in North America (Fig. 3(b)). Glufosinate is also widely used in South America, especially due to the large adoption of no-till cropping systems (e.g. glufosinate-resistant cotton in northeast Brazil).[10] Rice, orchards, vineyards, minor crops and non-agricultural areas also represent a large portion of glufosinate use in western United States and other parts of the world. Glufosinate used to be registered for use in Europe until 2018, but it has not been reapproved since then by the European Commission due to toxicology concerns.

Glufosinate use will likely continue to increase in the near future for a number of reasons. First, glyphosate-resistance is widespread, and weeds continue to evolve multiple resistance to other postemergence herbicides such as protoporphyrinogen oxidase (PPO) inhibitors and synthetic auxins (2,4-D and dicamba), as well as metabolic resistance to key preemergence herbicides (e.g. *S*-metolachlor).[11] Furthermore, glufosinate is not considered volatile and the newer transgenic technologies for herbicide-resistant crops tend to stack multiple events, including glufosinate resistance as one of their traits in most varieties. Glufosinate will also continue playing an important role in tropical countries such as Brazil, where grasses are the biggest challenge for weed management[12] and paraquat has recently been banned. Lastly, while there has been an increased effort to develop new herbicide modes of action, no new effective and non-selective herbicide is expected to launch in the next few years.[13]

3 PHYSICOCHEMICAL PROPERTIES AND *IN PLANTA* BEHAVIOR

Glufosinate (molecular weight 181.13 g mol^{-1}) is a phosphinic acid belonging to the organophosphorus chemical family, unlike any other chemical class of herbicides.[4] Glufosinate is the most hydrophilic herbicide ($logK_{ow}$: −4.0), with different ionization constant values (pKa: 2, 2.9, and 9.8) as a result of the amine and hydroxyl groups present in its structure (Table 1). It is not

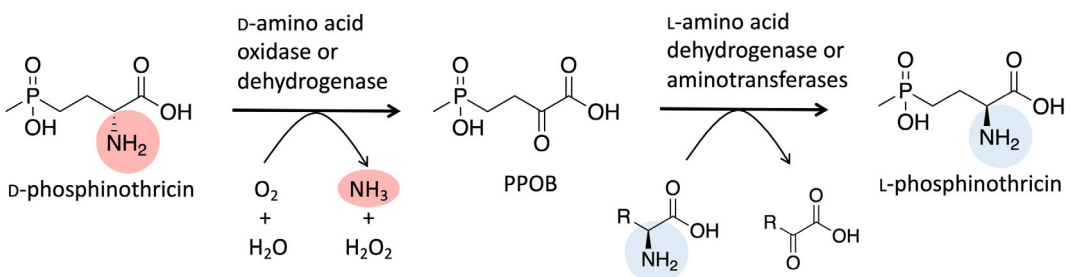

Figure 2 Method to convert D-phosphinothricin into L-phosphinothricin.[5] Both steps can be performed either enzymatically or chemically. Amino acids such as L-glutamate, L-glutamine, L-lysine, L-alanine, and L-phenylalanine work as the amino group donor in the second step. PPOB: 4-methylphosphinico-2-oxo-butanoic acid.

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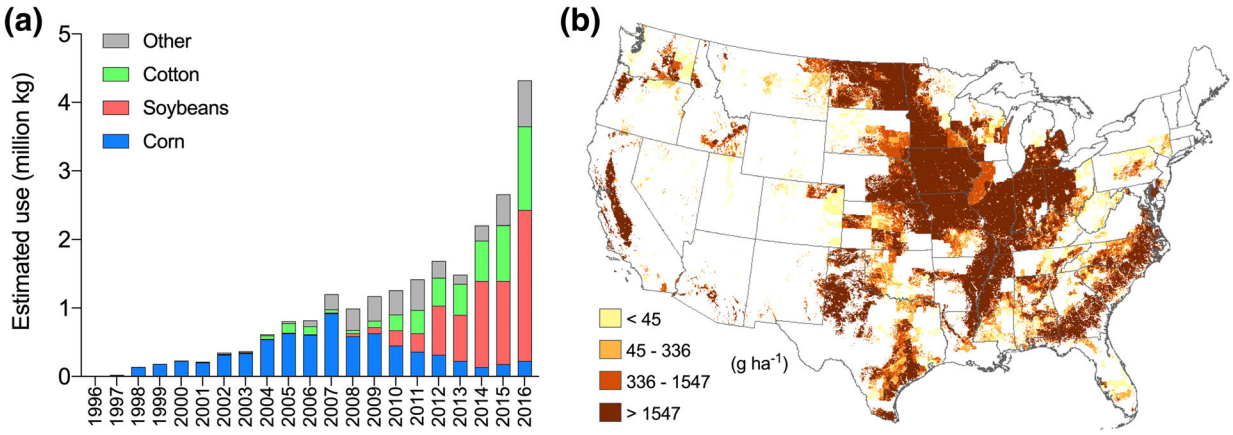

Figure 3 Glufosinate use in the United States has exponentially increased over the last decade and is intensively used in the Midwest and Southern regions where glufosinate-resistant crops are grown. Estimated use by year and crop from 1996 to 2016 in the United States (a). The category 'other' includes vineyards, orchards, wheat, rice, pasture, vegetables, and non-agricultural areas. Estimated agricultural use for glufosinate across the United States in 2016 (b). Source: United States Geological Survey (USGS). Accessed in May 4, 2020. Available at: https://water.usgs.gov.

considered volatile (vapor pressure: 10^{-4} Pa) and available formulations are typically the ammonium salt, which tends to dissociate in aqueous solution.[14]

There are a number of factors affecting the uptake and translocation of glufosinate in plants.[15–17] For instance, glufosinate uptake may be driven by an active transporter under low concentrations, but at normal field rates, absorption occurs mostly by cell to cell diffusion, that is, absorption is dependent on glufosinate concentration gradient between the inside and outside of cells.[15] In addition, the presence of glutamine strongly decreases glufosinate absorption, suggesting that glufosinate and glutamine may compete for the same active transporter. Glufosinate translocation throughout the plant relies mostly on apoplastic translocation in the xylem, which depends on transpiration rates. Consequently, glufosinate molecules tend to accumulate in older leaves with higher transpiration rates as opposed to younger leaves and apical meristems.[18] The fast action of glufosinate does not limit its own translocation. If that was the case, D-phosphinothricin (does not bind to GS) would translocate more efficiently than L-phosphinothricin or the racemic mixture, but poor translocation in plants has also been observed for the L-isomer.[19]

A comparative study between glufosinate and glyphosate translocation using artificial membranes demonstrated that both herbicides are unlikely to move across lipophilic layers due to their high hydrophilicity (glyphosate logK_{ow}: −3.1).[20] However, unlike glufosinate, glyphosate can easily translocate to different parts of the plant, especially from source to sink organs with the movement of photosynthates. This supports the hypothesis that glyphosate possesses an effective active transporter to cross lipophilic cellular membranes.[21] Indeed, glyphosate uptake was inhibited in the presence of phosphate, suggesting that they may compete for the same transporter. If the fast action of glufosinate does not self-limit translocation, the contact activity of glufosinate probably results from the combination of its physicochemical characteristics and the lack of an effective active transporter.[15]

4 GLUTAMINE SYNTHETASE, THE TARGET OF GLUFOSINATE

Glufosinate targets GS, the second most abundant protein in plant leaves, that is essential for nitrogen metabolism by catalyzing the adenosine triphosphate (ATP)-dependent incorporation of ammonia into glutamate to yield glutamine (Fig. 4).[22,23] GS works in a two-step reaction, in which glutamate is first phosphorylated into γ-glutamyl-phosphate using ATP, followed by ammonia assimilation yielding glutamine and releasing phosphate. Several end products of glutamine metabolism can allosterically regulate GS activity: serine, alanine, glycine, adenosine monophosphate, cytidine triphosphate, tryptophan, histidine, carbamoyl phosphate, and glucosamine-6-phosphate.[24] Each one of these inhibitors leads to partially inhibition of GS but can completely block enzyme activity when collectively.

Once ammonia is incorporated into glutamate by GS, glutamine and 2-oxoglutarate can then be converted back into two glutamate molecules by glutamine 2-oxoglutarate aminotransferase (GOGAT) (Fig. 5). The two enzymes (GS/GOGAT) work in concert

Table 1 Glufosinate and its metabolite are highly water soluble. Physicochemical properties for glufosinate and its main metabolite MPP (3-[hydroxy(methyl)phosphinyl]propionic acid)

Property		Glufosinate	MPP
Vapor pressure (20 °C)		No volatility	No volatility
Solubility in water		1370 g L^{-1}	794 g L^{-1}
	pH 5	>500 g L^{-1}	—
Solubility in buffer	pH 7	>500 g L^{-1}	—
	pH 9	>500 g L^{-1}	—
Partition coefficient (K_{ow})	pH 7	<0.1	0.06

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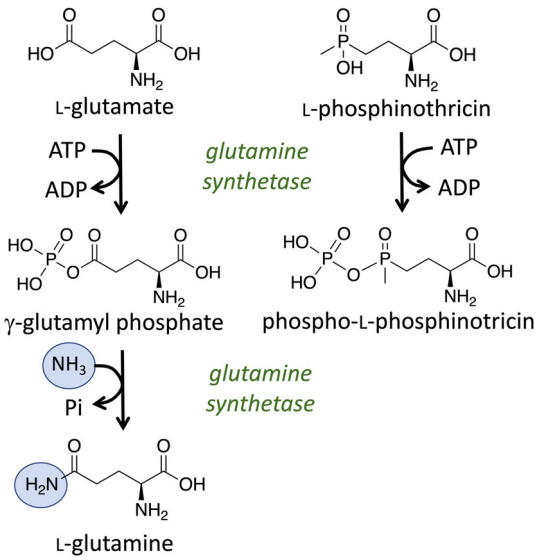

Figure 4 Glufosinate (L-phosphinothricin) inhibits the two-step reaction catalyzed by glutamine synthetase (GS). In the absence of the inhibitor, GS phosphorylates glutamate into γ-glutamyl-phosphate and incorporates ammonia to yield glutamine. Glufosinate competes with glutamate for the active site. The enzyme phosphorylates glufosinate into phospho-L-phosphinothricin, but ammonia cannot be incorporated in the second step. This leads to the formation of an irreversible enzyme-inhibitor complex.

to form the main route for ammonia assimilation into nitrogen organic compounds.[22] Plants have two main isoforms: GS1, which is located in the cytoplasm, and GS2 functioning in the chloroplast.[25] Because glutamine can donate an amino group to other organic compounds in the plant, GS1 is essential for nitrogen export throughout the plant in form of glutamine.[23] In contrast, GS2 plays a key role recycling ammonium from the photorespiration pathway.[26] Plants can also take up nitrogen in the form of both ammonium or nitrate. In the cytosol, nitrate reductase converts nitrate into nitrite, which is then converted into ammonium by nitrite reductase before it can be incorporated into amino acids by GS.[27]

In green leaves of C3 plants, GS2 is the predominant isoenzyme, but for some C4 and CAM (crassulacean acid metabolism) species, the contribution of GS1 to the total GS enzyme activity can reach up to 80%.[28] Loss of function mutants have been described in *Arabidopsis* and barley for GS2 and GOGAT.[29, 30] These mutants are unable to grow under normal air (400 ppm CO_2; 21% O_2), but they develop normally under non-photorespiratory conditions (1000 ppm; 2% CO_2), demonstrating the importance of the GS/GOGAT pathway to photorespiration. In C4 species, while GS2 has a greater contribution to the total GS activity in bundle sheath, GS1 is predominant in mesophyll cells.[31] In addition, both isoforms are slightly more tolerant to glufosinate in the bundle sheath cells than in the mesophyll, and GS2 less sensitive than GS1.[31]

Glufosinate is an irreversible inhibitor of both GS1 and GS2 with an inhibitor dissociation constant (K_i) between 1.1 and 4.8 μmol L^{-1}, depending on the plant species.[32] It is a competitive inhibitor with respect of glutamate for the active site, and uncompetitive with the other substrates (ATP and ammonia). This is because phosphorylated L-phosphinothricin (phospho-L-phosphinothricin) shares a high level of structural similarities with γ-glutamyl-phosphate (phosphorylated glutamate). In addition to glufosinate, at least 13 glutamate analogs have been reported as GS inhibitors. Methionine sulfoximine is also a strong inhibitor of GS and, perhaps, the second most studied among them. In a comparison study, glufosinate was 2.2 times more powerful than methionine sulfoximine inhibiting both plant and bacterial GS.[33] Unlike glutamate competitors, the analogs of 3,5-dichlorophenylaminomethylenebisphosphonic acid display a different mechanism of GS inhibition. A kinetic characterization in maize GS showed that these phosphonic acids have a non-competitive mechanism against glutamate and an uncompetitive mechanism against ATP.[34] Despite the numerous compounds with potential

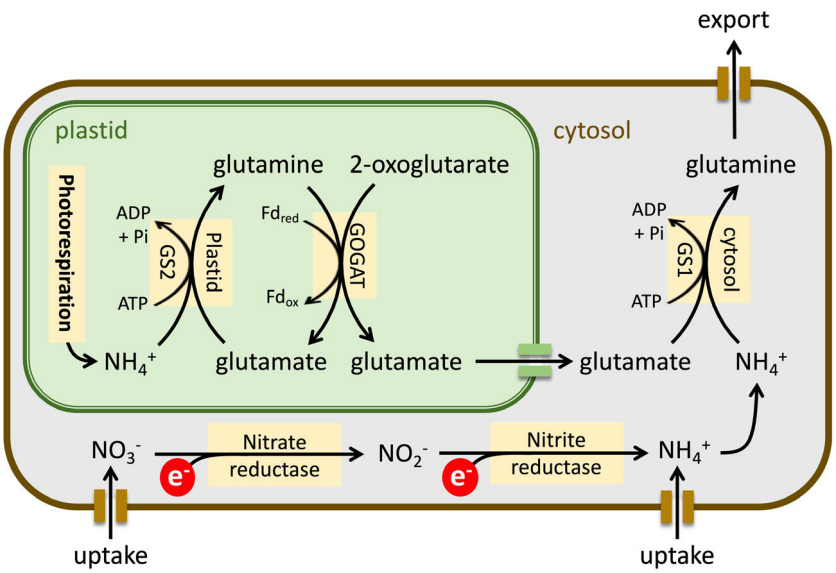

Figure 5 The GS/GOGAT pathway is the principal mechanism of primary and secondary nitrogen assimilation into organic compounds in plants. Plants can uptake nitrogen in form of nitrate or ammonium, which are incorporated into glutamine by the cytosolic glutamine synthetase (GS1) and exported to other parts of the plant. In the chloroplast, GS2 recycles ammonium produced by the photorespiration pathway. Glutamate-oxoglutarate aminotransferase (GOGAT) yields two molecules of glutamate out of glutamine and 2-oxoglutarate.

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for GS inhibition, glufosinate is the only one that has been developed as a herbicide, possibly due to its stronger capacity to inhibit GS in plants.

The inhibitory mechanism of glufosinate has been characterized in the crystal structure of maize GS1.[35] A homology model of *Amaranthus palmeri* GS2 revealed that glufosinate interacts with exactly the same residues as in maize (Fig. 6). Glufosinate binding involves H-bond interactions with Glu131, Glu192, Arg332, Gly245, His249, Arg291 and Arg311, as well interactions between the phosphate group and manganese atoms. The binding of ATP in the catalytic domain involves hydrogen-bond interactions with Glu129, Ser187, Ser253, Arg311, Arg316 and Glu330, as well as π–π interactions between the adenosine ring with Tyr328 and interactions between the diphosphate groups and manganese atoms. Residues Glu297 and Asp56 are conserved among several plant GSs and play a role as a flap to guard the substrate glutamate entrance to the active site and to bind ammonium substrate, respectively.[36] In addition, His249 was identified by site-directed mutagenesis as a key residue for the differential stability of the substrates on GS.[35] Changing amino acids in these positions or in allosteric sites would likely affect the binding of glufosinate as well as glutamate.

5 GLUFOSINATE TOXICOLOGY

The target of glufosinate, GS, is present both in plants and animals. Consequently, the toxicity of the active ingredient has been extensively studied.[37] Exposure to high levels of glufosinate can cause a reversible reduction in glutamine concentrations in mammalian tissues. However, there is no evidence for accumulation of glufosinate in mammals, as it is a highly water soluble compound.[38] The acute toxicity of both oral and dermal exposures to glufosinate were very low in rats and slightly higher in mice (Supporting Information Table S1). This difference is probably associated with divergences in kinetics and bioavailability between these species. There was also no sex-specific differences from any route of administration.[1]

In the same study, glufosinate was non-sensitizing to dermal or eye exposure, but was moderately irritating to the eyes due to some of the adjuvants in the formulation. In contrast, glufosinate exposure to eyes increased intralocular pressure in mice suggesting that the herbicide could damage the optic nerves when improperly handled.[39] The sub-chronic feeding studies of glufosinate were carried out over 3 months at various concentrations using rats (up to 4000 mg kg^{-1} diet), mice (up to 1280 mg kg^{-1} diet), and dogs (up to 256 mg kg^{-1} diet).[40] The 'no observed adverse effect levels' were 2–4 mg kg^{-1} d^{-1} for dogs, 4.1 for rats and 17 for mice. A chronic toxicity and oncogenicity study showed that at very high concentrations (between 140 and 500 mg kg^{-1} diet), only slight effects were observed on mortality of female rats, kidney weights, anemia in both sexes, GS activity in liver and kidneys, and glutathione level in liver and blood.[40] There was no glufosinate-related non-neoplastic or neoplastic lesions, resulting in 'no observed adverse effect levels' of 40 mg kg^{-1} diet.

Exposing mice to glufosinate prenatally reduced locomotor activity, impaired memory formation and autism-like behaviors. This was associated to a significant alteration in gut microbiome, which could be the cause of the behavioral abnormalities.[41] Another study reported no effect on body-weight gains in the parent or offspring, nor on the survival of the offspring.[38] Another factor contributing to the safety of glufosinate on animal systems is the rapid rate of metabolic degradation of the active ingredient,

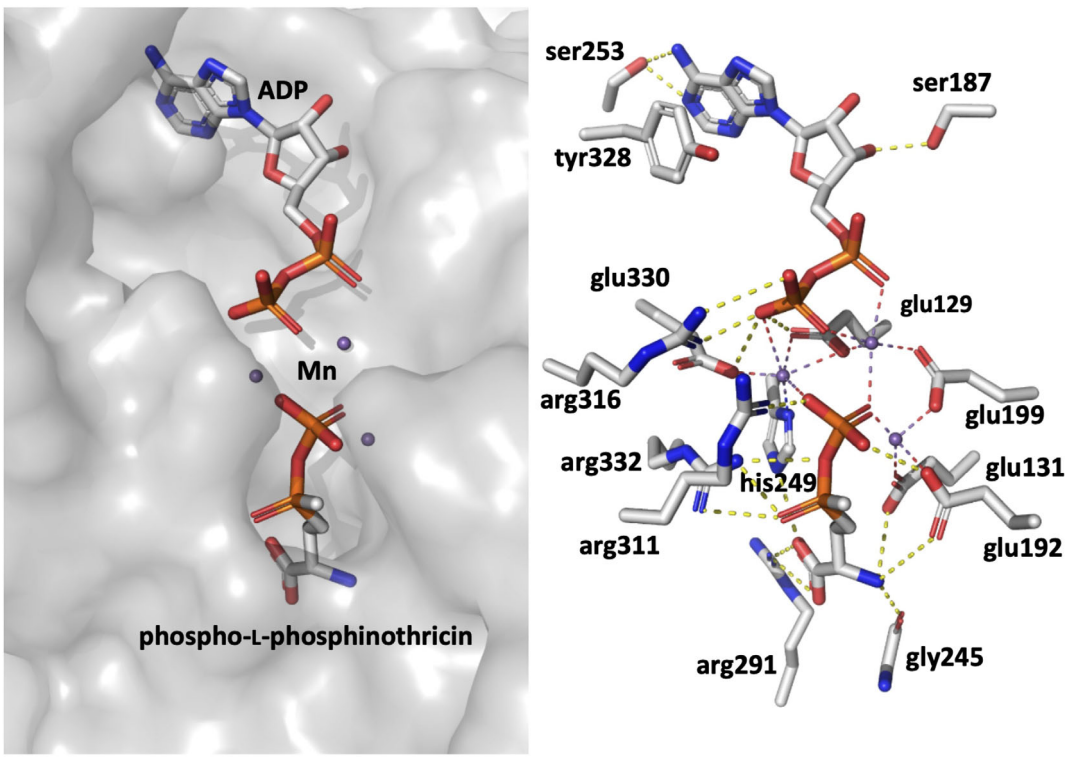

Figure 6 The catalytic domain of glutamine synthetase (GS) on the left, and the amino acid residues interacting with glufosinate and adenosine diphosphate (ADP) on the right.[35] Glufosinate is phosphorylated (phospho-L-phosphinothricin) by adenosine triphosphate (ATP) and irreversibly binds to GS. Three manganese (purple spheres) ions work as cofactors and help to stabilize the transfer of phosphate from ATP to glufosinate.

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with most of the product and/or its metabolites excreted through urine and feces. Since the toxicological data indicated no genotoxic, carcinogenic, or teratogenic potential or other special toxicological hazards, a safety factor of 100 was sufficiently conservative to establish an acceptable daily intake value of 0.02 mg glufosinate kg^{-1} d^{-1}.[40] In contrast, a retrospective cohort study conducted with 253 patients for almost 20 years indicated 13% of mortality due to acute poisoning resulting from improper handling of the herbicide. The causes of death were associated with decreased Glasgow coma scale and bicarbonate, use of mechanical ventilator, and vasopressors.[42] Therefore, the ingestion of a large amount of glufosinate by humans can cause intoxication problems, but the proper use of glufosinate as a herbicide can be considered safe when guidelines are followed.[43]

6 GLUFOSINATE IN THE ECOSYSTEM

The increased use of glufosinate in the recent years results from a larger treated area with the herbicide rather than higher rates per area. Currently, the maximum application rate for the active ingredient is 1470 g ha^{-1} yr^{-1} although most farmers perform only one post-emergence application at 420–655 g ha^{-1}, depending on the crop. Therefore, glufosinate residue levels in the food chain are not expected to rise as glufosinate use increases. A study on glufosinate metabolism and translocation in transgenic glufosinate-resistant crops concluded that postemergence applications (670 g ha^{-1}) pose minimal risk to the consumer. This was supported by the fact that most of the applied herbicide was lost by precipitation and microbial activity. The remaining residue in the plant reach the soil when the leaves fall off before harvesting. In addition, glufosinate applications in the field are normally performed early in the season, providing enough time for residues to dissipate. The final glufosinate concentration in the grain was less than 0.1 mg kg^{-1} (< 0.3% of the total applied).[44] Alternatively, switching from the racemate to the single enantiomer could reduce by half the total amount of glufosinate applied to the environment. A recent study has shown that L-phosphinothricin was favored in an efficacy-risk assessment by better inhibiting weed growth and less early life toxicity to off-target organisms compared to the racemic mixture.[45] Since glufosinate and its metabolites are highly water soluble, they neither volatilize nor accumulate in the food chain (Table 1).[1] Glufosinate does not remain in the soil because it is rapidly degraded by soil bacteria, resulting in no residual activity nor crop rotation restrictions.[46] Glufosinate persistence in the environment was the shortest compared to four other herbicides (glyphosate, phenmedipham, ethofumesate and metamitron), and no indication for potential groundwater pollution risk was observed.[47]

Dissipation of glufosinate in the soil occurs primarily via oxidation, transamination, and acetylation reactions by soil microbes. The half-life of glufosinate in the field varies between 1 and 4 days, but it can be substantially increased in sterile soils.[46] In addition, L-phosphinothricin was preferentially degraded as opposed to the D-isomer, and both enantiomers were considered stable in water.[48] Glufosinate oxidation is irreversible and ultimately results in the release of carbon dioxide. Glufosinate can be susceptible to leaching from the soil surface a few hours after spraying.[49] However, organic residues are often present within the top 20 cm, which ensures complete dissipation of glufosinate before it can be transported to lower layers in the soil profile.[50]

Glufosinate provides minimal concern on aquatic ecosystems. In fact, most effects reported are due to some of the adjuvants in the commercial formulation and not associated with the active ingredient.[50] Glufosinate-based inhibition of GS in plants may lead to a transient release of ammonium into the soil and atmosphere, and a subsequent short-lived stimulation of oxygen consumption in the soil.[51] However, its rapid metabolic degradation helps to prevent meaningful exposure to earthworms, beneficial arthropods, bees, and mammals.[50] Using newer microbe sequencing technologies, a study concluded that glufosinate application had no adverse effects on the rhizosphere bacterial community composed by a wide variety of phyla, including proteobacteria, bacteroidetes, acidobacteria, gemmatimonadetes and actinobacteria.[52]

In summary, glufosinate reaching the soil surface following postemergence application is rapidly metabolized by soil microbes and dissipates to trace amounts within 1–7 days after treatment, depending on environmental conditions and soil characteristics. Both glufosinate and its metabolites do not volatilize nor accumulate in the fatty tissue of fish or other animals. For this reason, there is no concern of bioaccumulation of residues in the environment. The toxic threshold levels for all tested representatives of non-target organisms are extremely higher than the expected worst-case environmental concentrations that no harmful effects can occur. Regardless of the above, proper pesticide management of any kind are always encouraged to mitigate potential risks for both the environment and human health.

7 MODE OF ACTION IN PLANTS

7.1 Relationship between GS inhibition, ammonia accumulation and carbon assimilation

The mode of action (MoA) of glufosinate has been controversial for many years. While glufosinate is a potent inhibitor of GS, the rapid phytotoxicity does not result from a reduction in glutamine pool levels. If amino acid depletion was the phytotoxic factor, plants would likely show a slow response to the herbicide similar to what is observed for glyphosate and acetolactate synthase inhibitors.[2] In contrast, susceptible plants develop foliar injury within a few hours after glufosinate treatment. Inhibition of GS causes a dramatic accumulation of ammonia, and this has been proposed as the main driver for the fast response to glufosinate (Fig. 7). Previous studies have demonstrated that more than 60% of the total accumulated ammonia comes from photorespiration.[53] In fact, evidence shows that the catastrophic consequences of GS inhibition by glufosinate is associated with reduced capacity to cope with photorespiration.[54,55]

In addition to the accumulation of ammonia, glufosinate can also inhibit carbon assimilation.[56,57] The effect of glufosinate on carbon assimilation was stronger at 21% O_2 and 400 ppm CO_2 (photorespiratory conditions) compared to 2% O_2 and 1000 ppm CO_2 (non-photorespiratory conditions).[54] Under photorespiratory conditions, plants cannot reach their compensation point because rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) uses a significant amount of O_2 relative to CO_2. Furthermore, C3 plants generally show stronger inhibition of carbon assimilation than C4 plants.[54] This is because C4 plants have a modified bundle sheath cells that concentrates CO_2 in the mesophyll where rubisco is located. These findings indicate that photorespiration is connected to carbon assimilation inhibition by glufosinate.[56]

While ammonia accumulation is a physiological consequence of GS inhibition, several reasons indicate that this is not the main cause of phytotoxicity. Ammonia toxicity causes severe chlorosis of leaves, suppression of growth, and eventually plant death,[8]

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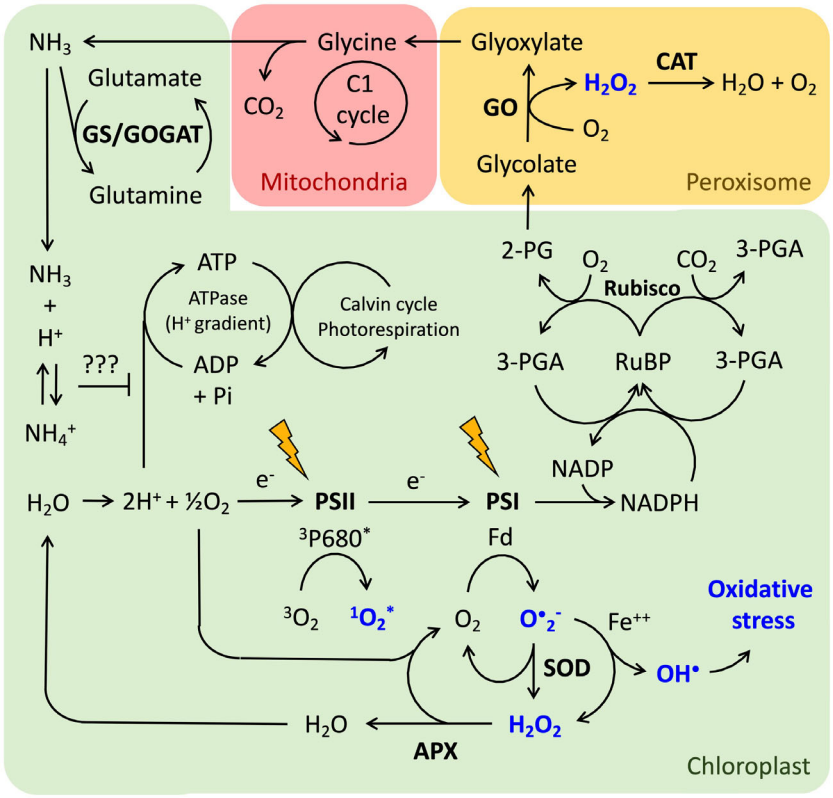

Figure 7 The mode of action of glufosinate. Inhibition of GS disrupts Rubisco's carboxylase and oxygenase activity, a major sink for chemical energy (NADPH and ATP) produced in the light reactions of photosynthesis. The excess of electrons overwhelms the antioxidant system (water–water cycle) and is accepted by molecular oxygen leading to oxidative stress and rapid cell death. Reactive oxygen species (ROS) generation sites are highlighted in blue. NADPH, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate; 2-PG, phosphoglycolate; 3-PGA, phosphoglycerate; APX, ascorbate peroxidase; CAT, catalase; Fd, ferredoxin; GO, glycolate oxidase; GOGAT, glutamate oxoglutarate aminotransferase; GS, glutamine synthetase; PS, photosystem; Rubisco, ribulose-1,5-biphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-biphosphate; SOD, superoxide dismutase.

which differ from the rapid necrosis observed with glufosinate treatment. Ammonia accumulated to high levels in glufosinate-treated plants exogenously supplied with glutamine and in glufosinate-treated plants under low light, but no apparent rapid phytotoxicity was observed in either treatment.[15,58,59] A physiological comparison between mature and young leaves demonstrated that ammonia accumulated in both leaf types, but rapid phytotoxicity was observed only in older leaves.[58] Likewise, the addition of low doses of atrazine to glufosinate prevented the rapid phytotoxicity, but ammonia still accumulated to high levels in these plants.[59] Ammonia and other amines have been proposed as uncouplers of photophosphorylation in the light reactions of photosynthesis, ultimately decreasing carbon assimilation rates.[60] Photosynthetic uncouplers disrupt the coupling between the electron transport and phosphorylation reactions and thus inhibit ATP synthesis without affecting the respiratory chain and ATP synthase.[61] However, while adding ammonia to chloroplasts decreased the formation of ATP, no inhibition of ferricyanide reduction was observed, nor electron flow inhibition.[60] Finally, ammonia accumulation under non-photorespiratory conditions (high CO_2 and low O_2) did not cause inhibition of carbon assimilation.[53] In addition, carbon assimilation inhibition did not correlate with ammonia accumulation nor the development of symptoms in different weeds.[58] In fact, the early studies reporting inhibition of carbon assimilation by glufosinate treatment never mentioned such a correlation with phytotoxicity.[53–55] Therefore, ammonia accumulation to high levels can be toxic to plants, which may contribute only in part for glufosinate herbicidal activity. However, ammonia accumulation is not consistent with the rapid development of symptoms and cannot explain *per se* the mode of action of glufosinate.

7.2 The role of reactive oxygen species

A recent breakthrough research challenged the long-accepted paradigm regarding the mode of action of glufosinate.[58, 59] There is now strong evidence that reactive oxygen species (ROS) are the main driver for the rapid phytotoxicity observed in glufosinate-treated plants. The massive light-dependent production of ROS causes the catastrophic lipid peroxidation of the cell membranes, and consequently, rapid cell death. This was supported by the glufosinate-induced accumulation of malondialdehyde, a byproduct of oxidative stress through lipid peroxidation.

Based on a detailed series of investigations, a new model has now been proposed to illustrate the relationship between GS inhibition and ROS accumulation (Fig. 7).[59] First, glufosinate inhibits GS, leading to glutamine and glutamate depletion, and photorespiratory ammonia accumulation. Consequently, glyoxylate is not converted into glycine by the glutamate-dependent aminotransferase, which leads to glycolate and glyoxylate accumulation.[55,59] This leads to a feedback inhibition of the photorespiration pathway and subsequent inactivation of the Calvin cycle.[62] If both photorespiration and the Calvin cycle are disrupted, the equilibrium between energy generation and consumption is broken, leading to a massive oxidative stress in the chloroplasts. Under these conditions, the photosystems are still able to capture energy from the sunlight to

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maintain the flow of electrons, but nicotinamide adenine dinucleotide phosphate (NADPH) and ATP are not being consumed. Consequently, the excess of electrons is accepted by molecular oxygen, leading to ROS accumulation, especially under full sunlight. The massive buildup of ROS in the chloroplast thylakoids leads to the catastrophic consequences of cell membrane peroxidation.[58,63] A simpler alternative model is based on the hypothesis that glufosinate could play a second role as an uncoupler through either ammonia accumulation or another physiological factor.[64] In fact, plants accumulated higher levels of ROS when sprayed with glufosinate plus a low dose of dinoseb (a known uncoupler of photosynthesis) compared to glufosinate only.[59] However, the uncoupling activity of glufosinate and its physiological consequences remains to be proven.

Regardless the mechanism by which ROS are formed, the rapid phytotoxicity in glufosinate-treated plants originates from a massive light-dependent oxidative stress. Plants react to the nascent ROS-driven oxidative stress by increasing the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase following glufosinate treatment.[59, 65] In addition, glufosinate treatment induced the expression of senescence-related transcription factors, involved in other ROS-driven processes such as self-destruction and programmed cell death[66] Thus, glufosinate disrupts the balance between generation and scavenging of ROS. Hydrogen peroxide is partially generated by the glycolate oxidase activity in the peroxisome (Fig. 7). Inhibition of GS disrupts the photorespiration pathway and linear electron flow in the light reactions of photosynthesis. When plants are treated at full sunlight, the antioxidant system is overwhelmed, and electrons are then accepted by O_2 coming from the breakdown of water in photosystem II. The subsequent ROS accumulation leads to a catastrophic lipid peroxidation and forms the basis for the fast action of glufosinate.[58, 59]

8 TRANSGENIC CROPS FOR GLUFOSINATE RESISTANCE

The first glufosinate-resistant crop was developed for canola in 1995, followed by corn in 1997, cotton in 2004, and soybean in 2011. Other crops such as rice, sugar beet and wheat have also been genetically modified for glufosinate resistance but are not currently available for agricultural use. Glufosinate-resistant crops can metabolize glufosinate by expressing the *phosphinothricin acetyltransferase* (*pat*) gene, which is also known as *bialaphos resistance* (*bar*) gene. These plants rapidly convert L-phosphinothricin into *N*-acetyl- L-phosphinothricin, a non-phytotoxic metabolite of glufosinate (Fig. 8). Expression of *pat* or *bar* at high levels allows the application of glufosinate postemergence in glufosinate-resistant crops such as soybean, cotton, corn and canola.[67] Other methods to obtain glufosinate resistant plants have been reported such as GS overexpression and mutations,[36,68,69] but only the *pat* and *bar* genes have been used to develop glufosinate-resistant crops.

In addition to glufosinate-resistant crops, the *pat* and *bar* genes have been employed as selectable markers for countless plant transformation protocols. Transformed plants with those genes can survive glufosinate treatment, providing an excellent system to screen a large number of plants with a single treatment. For those reasons, glufosinate plays an essential role in plant biochemistry, genetic regulation, and molecular biology research. For instance, transgenic cotton cultivars expressing *cry* genes for insect resistance were developed using the *pat* gene as a selectable marker. These commercial cultivars retain low levels of *pat* expression providing crop tolerance to field rates of glufosinate. Consequently, farmers have often been applying glufosinate to these cotton cultivars for postemergence control of weeds, especially for managing glyphosate-resistant *Amaranthus palmeri*. However, the low messenger RNA (mRNA) expression level of the *pat* gene in those cultivars does not provide the same level of protection against glufosinate as of that achieved with the high expression of the *bar* gene in glufosinate-resistant cotton.[67] Therefore, even though this practice has been widely adopted, farmers should avoid using glufosinate on cotton cultivars not expressly designed as resistant to this herbicide because the safety margin to glufosinate is considerably lower.

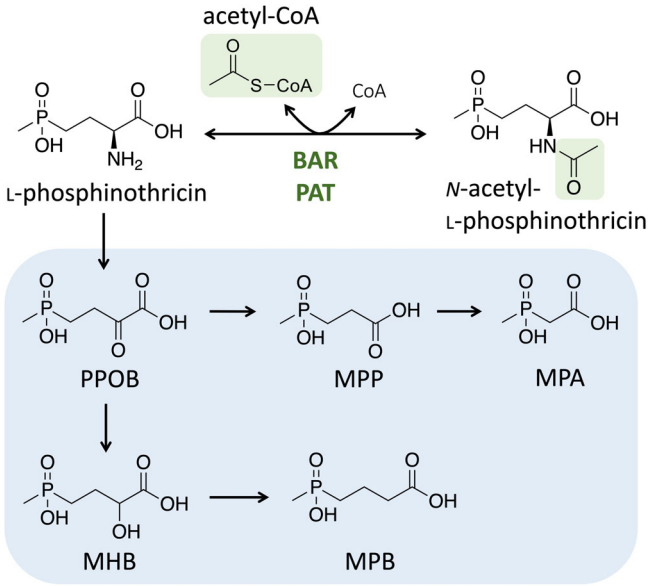

Figure 8 Metabolism of L-phosphinothricin into *N*-acetyl- L-phosphinothricin by bialaphos resistance (*bar*) or phosphinothricin acetyltransferase (*pat*) genes in glufosinate-resistant crops (green). D-Phosphinothricin cannot be metabolized by these genes. Metabolism of L-phosphinothricin in non-transgenic plants (blue). PPOB, 4-methylphosphinico-2-oxobutanoic acid; MPP, 3-(hydroxymethylphosphinyl)propionic acid; MPA, 2-methylphosphinicoacetic acid; MHB, 4-methylphosphinico-2-hydroxybutanoic acid; MPB, 4-methylphosphinicobutanoic acid.[44]

9 GLUFOSINATE METABOLISM IN WEEDS

Non-transgenic plants can convert glufosinate into five main metabolites: PPOB; 3-(hydroxymethylphosphinyl) propionic acid (MPP); 2-methylphosphinicoacetic acid (MPA); 4-methylphosphinico-2-hydroxybutanoic acid (MHB); 4-methylphosphinicobutanoic acid (MPB) (Fig. 8). While there have been a number of studies investigating glufosinate metabolism in different species, the conclusions are not always consistent among them. Most of the older literature report extremely low rates of glufosinate metabolism, considering the parent molecule fairly stable across most weed species.[70] For instance, in a study with 20 different weed species, very low amounts of MPP and MHB were present in at least 14 species, and no other metabolites were detected.[71] In contrast, more recent publications report relatively high rates of glufosinate metabolism in several weed species (Table 2). Interestingly, several of those species are able to metabolize glufosinate at similar rates to that of glufosinate-resistant crops (e.g. soybean, cotton and corn) at 48 h after treatment (HAT). However, most of these weeds are not able survive glufosinate

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application at the field rate even though their herbicide metabolism rates are high. Differential metabolism of glufosinate across different species can contribute for plant survival in the field and potentially evolve as a mechanism of resistance to glufosinate.

10 DIFFERENTIAL SENSITIVITY TO GLUFOSINATE IN WEEDS

Glufosinate performance substantially differs across weed species in Supporting Information Table S2. For instance, the estimated dose (ED_{50}) to achieve 50% reduction in dry biomass can vary from 26 g ha^{-1} in *Erigeron canadensis* L. to 763 g ha^{-1} in *Lolium rigidum*. On average, grass species tend to be less susceptible to glufosinate than broadleaves, with a few exceptions such as *Chenopodium album*, which is not very sensitive to the herbicide. In addition, some weed species tend to develop symptoms faster than others, which may suggest the existence of different phytotoxic mechanisms or variable capacity to cope with ROS accumulation.[58] While there are several factors contributing to differential weed response to glufosinate, the amount of herbicide reaching the target site seems to be one of the most important. Glufosinate does not translocate well to meristematic organs, and monocot species tend to have multiple meristems in one single plant, which makes grasses generally more capable to survive than broadleaves. Glufosinate efficacy was proportional to the concentration of the herbicide in the leaf tissue of five different weeds (Fig. 9). For example, lower amounts of glufosinate were detected in tolerant weeds (*L. rigidum*) compared to sensible species (*Amaranthus palmeri*) after treatment with the same herbicide rate.[58] The levels of remaining glufosinate in the plant tissue depends on the ratio between uptake and metabolism rates by a particular weed species, which are both known to vary across different plants (Fig. 10). In addition, weed species have different morphologies, which also contribute to the differential interception of spray droplets, directly affecting glufosinate uptake.[77] Dicot species normally have horizontal broad leaves, which are more prone to intercept spray droplets compared to vertical narrow leaves in monocot weeds.

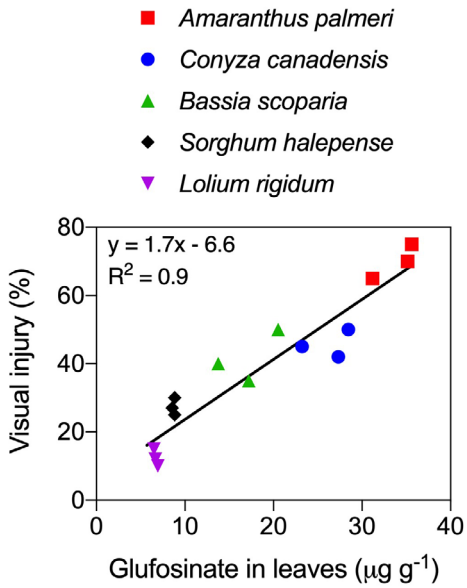

Figure 9 Glufosinate efficacy is proportional to the herbicide concentration in the leaf tissue. Positive correlation between glufosinate concentration within the leaf tissue and visual injury across four weed species. Data points represent different observation within each species.[58]

11 INCONSISTENT PERFORMANCE IN THE FIELD

Glufosinate is a contact herbicide with limited translocation making it effective primarily on annual weed species.[78] It also tends to provide lower activity on larger weeds compared to seedlings, and it is recommended to spray on small weeds (Fig. 10).[83] For example, glufosinate was more effective when applied at the 10-cm weed height compared to 15-cm in four different species.[78] Furthermore, a number of interspecific factors can decrease the efficacy of glufosinate (e.g. growth stage, leaf type and angle, cuticular waxes). More importantly, while glufosinate can work as an effective herbicide for glyphosate-resistant weeds, its field performance is strongly suppressed by inappropriate environmental conditions, particularly low temperature and low humidity around the time of application (Fig. 10).[16,84]

Table 2 High rates of glufosinate metabolism in weeds that are comparable to transgenic glufosinate-resistant crops. Glufosinate metabolism in crops and weeds at 48 h after treatment

Species[a]	Percent glufosinate (%)	Percent metabolites (%)[b]	Reference
T *Glycine max*	56	44	72
T *Gossypium hirsutum*	32	68	73
T *Zea mays*	61	39	74
Amaranthus palmeri	38–48	52–62	73, 75
Digitaria sanguinalis	43	57	74
Echinochloa crus-galli	47	53	75
Eleusine indica	40	60	74
Ipomoea lacunosa	75	25	73
Lolium perenne ssp. *multiflorum*	52	48	76
GR *Lolium perenne* ssp. *multiflorum*	21	79	76
Senna obtusifolia	51	49	74

[a]T, Transgenic glufosinate-resistant; GR, glufosinate-resistant.
[b]Metabolites are *N*-acetyl-L-phosphinothricin, 3-(hydroxymethylphosphinyl)propionic acid, and 4-methylphosphinico-2-hydroxybutanoic acid.

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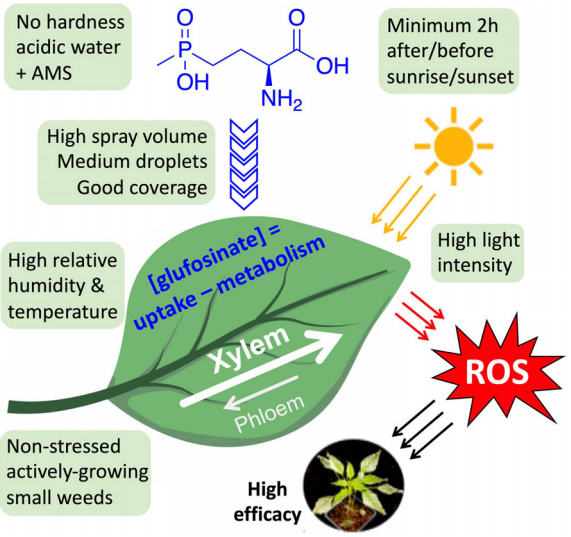

Figure 10 Glufosinate efficacy depends on several factors related to the spraying conditions, air temperature and humidity, and the target weeds. A good application technology and weather conditions can increase uptake levels, but the final concentration of glufosinate in leaves depends on metabolism rates. A greater concentration in the tissue will translate into increased ROS accumulation at full sunlight, ultimately leading to a better efficacy. AMS, ammonium sulfate; ROS, reactive oxygen species.[15,58,59,77–82]

There have been a number of studies attempting to determine the reasons for the variable responses of plant species to glufosinate. While leaf cuticular structure probably plays a role in the variable response of weed species to the herbicide, differential translocation is even more important. For instance, *L. rigidum* was significantly more tolerant to glufosinate compared to *Avena fatua*. The difference in sensitivity was consistent with a significant reduction in glufosinate translocation from treated leaves to the rest of the plant.[85]

Studies on the impact of relative humidity show that glufosinate efficacy is greater at higher humidity in the air. This is because under low humidity, herbicide droplets dry before sufficient glufosinate can be absorbed. There is some evidence suggesting that adding humectants to the spray solution can delay droplet dryness and increase glufosinate uptake, but this has not been used in the field.[84] Another study demonstrated that control levels of three *Amaranthus* species was higher at 90% humidity than at 35% humidity. This was correlated with a large difference in leaf uptake and glufosinate translocation out of the treated leaves.[16] Studies on temperature effects on control of weeds with glufosinate have consistently shown lower control under low temperatures. *Raphanus raphanistrum* control was greatly affected by temperature. Complete control could be achieved with 500 g ha^{-1} at 25 °C/20 °C, but the same rate of glufosinate provided less than 20% control at 10 °C/5 °C. This difference in sensitivity was correlated with an increase in the amount of glufosinate translocated out of the treated leaf at the higher temperature.[17]

12 TIME-OF-DAY EFFECT ON GLUFOSINATE EFFICACY

Because glufosinate is a light-dependent herbicide,[58] weed control also depends on the time of day of spraying (Fig. 10).[77] Early morning and late afternoon applications usually provide lower levels of weed control than midday applications. For example, glufosinate treatment at noon provided, on average, 40% higher levels of weed control compared to applications performed at 4 h after sunrise or before sunset.[79] Under greenhouse conditions, glufosinate application at 1 pm provided a substantially higher performance and consistency compared to 10 p.m. (Fig. 11(a)). The turnover of GS following glufosinate treatment at 1 PM *versus* at 10 p.m. provides interesting insights on why plants tend to survive applications in the dark (Fig. 11(b)). In the absence of light at the time of application (10 p.m.), plants are not affected by the massive ROS-driven burst since it is a light-dependent process. Consequently, plants are able to recover GS activity within 24 HAT, allowing survival and regrowth in the following days. Interestingly, higher levels of glufosinate uptake are observed in dark-treated plants compared to light-treated plants. However, higher glufosinate concentrations in the leaf tissue of dark-treated plants does not translate into higher phytotoxicity levels. This suggests when applications are followed by a dark period (sunset), plants could compartmentalize glufosinate somewhere in the cell (e.g. vacuole, apoplast) where it can no longer bind to GS even after the sunrise in the next morning. For these reasons, glufosinate applications are typically recommended under full sunlight, warm temperatures, and high humidity (Fig. 10).

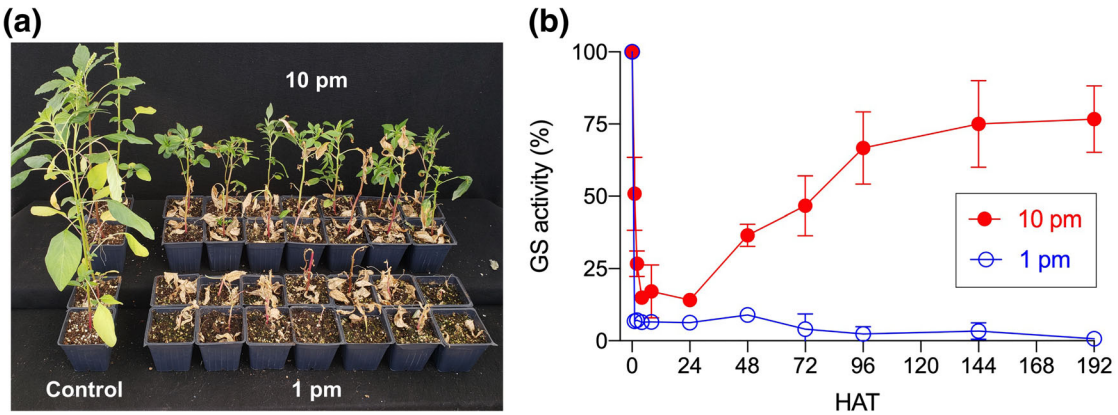

Figure 11 Glufosinate is a light-dependent herbicide and more effective when sprayed at full sunlight compared to night application. Time of day effect on glufosinate efficacy (a) and the turnover of glutamine synthetase (b) following glufosinate treatment at 1 or 10 p.m.

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13 OTHER FACTORS AFFECTING THE EFFICACY OF GLUFOSINATE

Several factors associated with the application conditions can also affect glufosinate efficacy (Fig. 10). Carrier water hardness and basic pH have shown antagonism for weak-acid herbicides such as glufosinate and glyphosate. Optimum levels of glufosinate efficacy on *Ambrosia trifida*, *Erigeron canadensis* and *Amaranthus palmeri* were obtained using an acidic carrier water (pH 4–6) that was free of hardness and metal cations such as iron, copper, and magnesium.[80] Ammonium sulfate is a well-known adjuvant for weak acid herbicides as it can increase uptake rates. While most glufosinate products are already formulated with surfactants, the addition of ammonium sulfate to the tank increases efficacy on most weed species significantly, and its use is widely recommended.[81] Finally, application technology is also an important factor limiting glufosinate efficacy and, applications are normally performed with spray volume higher than 140 L ha^{-1} using fine to medium size spray droplets, depending on the target weed species to achieve optimum coverage and performance. Though these conditions may change if glufosinate is sprayed in tank-mix with herbicides that are more prone to off-target movement such as the synthetic auxin herbicides, requiring larger spray droplet size.[82]

14 OPPORTUNITIES TO IMPROVE THE EFFICACY OF GLUFOSINATE

14.1 Synergism between glufosinate and protoporphyrinogen oxidase inhibitors

The new understanding on the mode of action of glufosinate has generated new ideas to enhance herbicidal activity. Since the rapid action of glufosinate is dependent on light activation of ROS, tank mixing it with other herbicides with similar physiological responses could lead to improved weed control. Herbicides targeting PPO cause protoporphyrin IX accumulation, which is a photodynamic compound that leads to ROS generation.[2] The tank mix of PPO-inhibitors and glufosinate provides enhanced herbicidal activity compared to the products applied individually.[86] The synergistic effect originates from the inhibition of GS by glufosinate leading to a transient accumulation of glutamate, the precursor for chlorophyll biosynthesis in plants. Inhibition of chlorophyll biosynthesis by PPO-inhibitors causes accumulation of protoporphyrin IX, which is enhanced by the accumulation of glutamate in the presence of glufosinate. Thus, the synergism observed with the mixture between glufosinate and PPO-inhibitors results from protoporphyrin IX accumulation to higher levels when both GS and PPO are inhibited simultaneously. The herbicide combination also provided improved weed control in the field, and may help to overcome negative effects of environmental conditions on glufosinate performance.[86] While this combination has potential to be widely adopted, the effect seems to vary across species, doses, and PPO chemistry. Thus, more research is needed to identify the best combination for each particular situation to maximize the synergistic effect on weed control.

14.2 Glufosinate interaction with other herbicides and inhibitors

Synergistic herbicidal compositions have also been reported for the combination of glufosinate and sulfonylurea herbicides.[87] Tank-mixing glufosinate with 2,4-D or dicamba also provided additive or synergistic effect controlling glyphosate-resistant broadleaf weeds and might be largely adopted in transgenic glufosinate + dicamba and glufosinate +2,4-D resistant crops.[88] However, glufosinate mixtures with other herbicides have not always worked well. For example, glufosinate antagonized acetyl CoA carboxylase inhibitors for *Eleusine indica* control.[89] Glufosinate also antagonized glyphosate efficacy on *Setaria faberi* and *Abutilon theophrasti* by reducing the translocation of glyphosate.[90] Glufosinate efficacy was reduced by adding monosodium methyl arsenate to the tank.[91] Therefore, glufosinate mixtures with other herbicides must be used carefully to minimize potential antagonistic effects and optimize herbicidal efficacy on weeds.

The use of compounds interfering with the plant capacity to detoxify ROS could also synergize glufosinate. For instance, dithiocarbamates chelators can suppress the activity of superoxide dismutase by removing copper from the enzyme. These compounds provided synergistic activity with paraquat as well as lactofen, herbicides generating ROS by different mechanisms.[92] However, the actual effect of dithiocarbamates as synergists of glufosinate has not been reported. Preliminary findings have recently suggested a potential enhancement of glufosinate efficacy by 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) under field conditions. The addition of 17 g ha^{-1} NBD-Cl to 656 g ha^{-1} glufosinate provided almost 100% control on 1 m-tall *Amaranthus palmeri* plants even when applications were performed in the dark (10 pm).[93] NBD-Cl can inhibit glutathione-*S*-transferases (GST) and has already proven to reverse metabolism-based herbicide resistance via GST conjugation.[94] Even though GST overexpression has been found in an *Amaranthus palmeri* population with reduced sensitivity to glufosinate,[95] the capacity for herbicide conjugation and its subsequent inhibition by NBD-Cl remains to be proven.

15 EVOLUTION OF GLUFOSINATE RESISTANCE IN WEEDS

To date, only five cases of glufosinate resistance in the field have been reported worldwide (Table 3).[11] Glufosinate resistance was first documented in a *Eleusine indica* field population from Malaysia, and the resistance mechanism in this population was not associated with target site alterations, herbicide metabolism, nor reduced uptake and translocation.[96] A potential glufosinate-resistance mechanism that has not been investigated in *Eleusine indica* is vacuolar sequestration, similar to what has been observed for glyphosate in *Erigeron* and *Lolium* species.[103] In addition, given that the herbicidal activity of glufosinate results from a massive oxidative stress, an increased antioxidant capacity could provide reduced sensitivity to glufosinate in plants, similar to other ROS-based herbicides such as PPO-inhibitors.[104] A *L. perenne* ssp. *multiflorum* population from Oregon showed enhanced levels of glufosinate metabolism (79%) compared to susceptible plants (48%), but the metabolites and enzymes involved are yet to be determined.[76] Other glufosinate-resistant *L. perenne* and *L. rigidum* populations have been identified, but their resistance mechanisms have not been investigated.[98,102] More recently, an *Amaranthus palmeri* population from Arkansas showed reduced sensitivity to glufosinate along with increased expression of genes associated to detoxification enzymes such as GST and cytochrome P450 monooxygenases.[95] However, the direct association between these enzymes, herbicide metabolism, and the lower sensitivity to glufosinate in *Amaranthus palmeri* requires further investigation.

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Table 3 A limited number of weed species have evolved resistance to glufosinate.[11] Cases of glufosinate resistance in the world and their respective resistance mechanism and multiple resistance profile

Species	Year	RF	Resistance mechanism	Multiple or cross-resistance	Reference
Eleusine indica	2009	11	Unknown	EPSPS/PSI/ACCase	96, 97
Lolium perenne	2015	6.2	Unknown	EPSPS/PPO/amitrole	98, 99
Lolium perenne ssp. *multiflorum*	2010	2.8	Metabolism/unknown	EPSPS	76, 100
	2015	5.5			
Lolium multiflorum	2017	11.8	Unknown	EPSPS/PPO	99, 101
Lolium rigidum	2017	7.4	Unknown	EPSPS/PPO	98, 99, 102

RF, resistance factor (based on lethal dose to achieve 50% reduction in growth or survival); EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase inhibitor; PSI, photosystem I inhibitors; ACCase, acetyl-CoA carboxylase inhibitors; PPO, protoporphyrinogen oxidase inhibitors.

In addition to increased metabolism rates conferring glufosinate resistance in *L. perenne* ssp. *multiflorum*, a target site mutation (Asp173Asn) had been associated with glufosinate resistance in a different population.[105] While this mutation segregated with the resistance phenotype, it was shown to be located far away from the catalytic site of GS.[76] A protein homology model of *L. perenne* ssp. *multiflorum* GS2 shows that Asp173Asn is located 19.8 and 18.1 Å away from glufosinate and ATP binding sites, respectively (Fig. 12). The mutation is positioned on a α-helix that is separated from the catalytic site by a β-sheet in between. The fact that GS is a highly allosteric enzyme suggests that even those mutations outside of the catalytic site could reduce glufosinate affinity. However, *in vitro* GS assays showed that the Asp173Asn mutation did not decrease the enzyme sensitivity to glufosinate, which proves that glufosinate resistance is not caused by that.[76] The assembled view of multiple GS chains forming a decamer further confirms that Asp173Asn is unlikely to affect the binding of glufosinate on its target site.

Amino acid substitutions affecting the residues that are critical for glufosinate stability in GS could potentially lead to a glufosinate-insensitive form of the enzyme (Fig. 6). For example, glufosinate-resistant soybean cells were obtained with an amino acid substitution (His249Tyr), providing 50-fold resistance at the cellular level.[69] *In vitro* enzyme activity assays showed that resistance in soybean cells was due to reduced GS sensitivity to herbicide inhibition. Amino acid alterations on residues Glu297 and Asp56 have also provided a glufosinate-insensitive GS by site-direct mutagenesis in dry beans.[36] While these target site mutations alter glufosinate binding to GS, they are also likely to affect the interaction of GS with glutamate, given the structural similarities between them. This would cause a considerable fitness penalty to resistant plants, given the essential role of GS for plant metabolism. In addition, artificial overexpression of GS provided glufosinate resistance to alfalfa and tobacco, and thus, could also evolve in weeds as a survival strategy.[68] Therefore, the evolution of target site resistance mechanisms leading to glufosinate resistance in weeds are possible as long as there is enough selection pressure by repeated use of this herbicide. Furthermore, the allosteric nature of GS increases the chances for the evolution of amino acid alterations leading to glufosinate resistance.

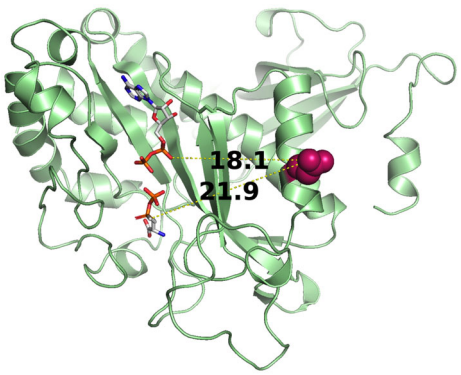

Figure 12 Previously reported target site mutation (Asp173Asn) does not provide glufosinate resistance. Structure of *Lolium perenne* ssp. *multiflorum* GS2 complexed with adenosine diphosphate (ADP) and phosphorylated glufosinate and its position relative to the location of the Asp173Asn mutation (red spheres). This mutation is between at least 18 Å away from that domain and unlikely to provide glufosinate resistance. This is also supported by *in vitro* enzyme assays.[76]

16 FINAL COMMENTS

In the light of the increasing number of herbicide resistant weeds, glufosinate plays an important role in weed management worldwide. With the development of genetically modified crops resistant to multiple herbicides, the use of glufosinate will likely continue to grow. While glufosinate performance can be affected by several factors associated to the weed species, the environment, and the application conditions, recent discoveries regarding how glufosinate works have paved the way for future research on different approaches to enhance the efficacy of glufosinate. The unique characteristics of glufosinate have intrigued scientists for many years and yet there is more to be discovered about this 'amino acid with unexpected herbicidal properties'.[1] The combination of glufosinate with PPO-inhibitors is one research area that requires additional studies to optimize the synergistic effect on different weed species and crops. Likewise, glufosinate resistance mechanisms require further investigation as the current understanding is not clear for most resistant populations. Lastly, even though glufosinate resistance has not evolved in many species, diverse strategies should be considered to preserve the efficacy of this herbicide and mitigate the evolution of multiple herbicide resistance.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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